SYNOPSIS

Lung is the organ of gaseous exchange. The epithelium of lung is exposed to inhaled air containing airborne pollutants, pathogens, particulate matters during the breathing process. Some of these chemicals are directly cytotoxic and some of them produce hazardous metabolic byproducts. Altogether these chemicals cause huge amount of redox burden on respiratory system. Lung epithelial cells must have unique repertoires to balance this redox burden or xenobiotic stress. In order understand the stress regulation in lung this study was initiated.

Integrated stress response (ISR) is a major pathway for cellular stress management in lung. There are four upstream kinases, GCN2, PERK, PKR and HRI. In presence of stress stimuli like, amino acid starvation, protein misfolding, double stranded DNA break or oxidative stress these kinases become phosphorylated and activate eIF2α. Activated or phosphorylated eIF2α falls off from the translation machinery to stop global translation. The mRNAs are stored in stress granules during this process and some stress regulator genes are activated by a non-canonical translation machinery. ATF4 is such a master regulator which gets activated and activate many other downstream genes.

According to previous studies FMRP was established as a multifunctional protein that helps in regulation of oxidative and genotoxic stress in the cells from different origin. In absence of this protein more oxidative stress in murine brain, perturbed DNA damage response during replication stress and perturbed stress granule biogenesis were reported. These reports suggested that FMRP may have an important role in cellular stress response pathways.

Expression of FMRP in lung epithelium and indication of FMRP involvement in cellular stress response, led us to investigate the role of this protein in the stress response of lung and lung derived cells.

The current study entitled “Role of Fragile X Mental Retardation Protein in Lung” is divided in to five chapters.

Chapter-I is the introduction, where literature reviews on FMRP and scope of the study has been described. **Chapter –II** describes the experimental procedures. A major part of this chapter has been published in the Journal of

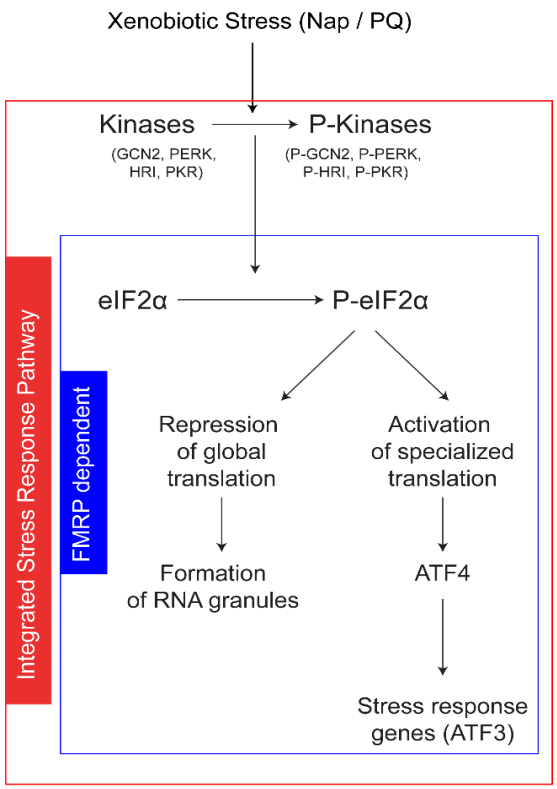
Cell Science. **Chapter –III** describes the study related to the protecting role of FMRP in lung and lung derived cell line. This chapter includes expression of FMRP and associated phenotype in murine lung, establishment of C22 cell line as an ex vivo model system, replicating FMRP phenotype in ex vivo model and role of FMRP in the Integrated Stress Response pathway. This chapter also talks about the role of FMRP in human lung. The dataset of this chapter has been published in the Journal of Cell Science. **Chapter-IV** deals with three alternative possibilities to explain FMRP phenotype in the lung. This chapter concludes that none of the known mechanisms like, FMRP dependent superoxide dismutase 1 translation, FMRP dependent activation of DNA damage response in replication stress or FMRP dependent stress granule biogenesis can sufficiently explain the susceptibility of FMRP deficient lung or lung derived cells to xenobiotic stress across species or stressors. The chapter also concludes that FMRP dependent actuation of ISR can fit as a plausible mechanism for the FMRP phenotype in the lung. A part of this chapter has been published in the Journal of Cell Science. **Chapter- V** is the discussion and future prospects of the study.

Chapter –III: The study was initiated to probe the role of FMRP in the regulation of stress response in the lung epithelial cells. This chapter deals with the major part of the study. The result section starts with the immunohistochemical analysis that shows expression of FMRP in murine lung epithelium, predominantly in the airway club and ciliated cells. It also shows that, in absence of FMRP there is no obvious histological difference in the lung tissue or any detectable difference in the expression of oxidative and genotoxic stress markers. Next, the animals were challenged with conventional dosage of naphthalene to create xenobiotic stress in the mouse lung. It was observed that in absence of FMRP airways had faster and greater club cell loss. It was also noticed higher level of oxidative and genotoxic stress marker expression and their longer persistence in the airway club cells. In order to investigate the cell autonomous function of FMRP in murine lung then naphthalene experiments were recapitulated in mouse club cell line, C22. The cell line experiments gave us opportunity to probe the time dependent events of cellular stress response more closely and mounting of ISR within 3 h of acute stress stimuli could be

observed. Here it was also noticed that in absence of FMRP, ISR was perturbed in the cells from very initial step of eIF2α phosphorylation. Knocking down ATF4 phenocopied FMRP, which led us to conclude that ISR is the major pathway of stress response during naphthalene induced stress and FMRP is involved in this process.

Next we went on to probe the role of FMRP in human lung. This section, describes expression of FMRP in human airway epithelium and a non-ciliated, bronchial cell line BEAS2B. The section also deals with the study of FMRP in BEAS2B cells during 9, 10-PQ injury. The study suggests that the protecting role of FMRP and its involvement in ISR is conserved in human bronchial cell line. It was that FMRP dependent activation of ISR is also applicable for the alveolar epithelial cell line A549 in response to PQ stress.

Overall the chapter concludes that FMRP is important for lung health, as it protects airway cells from xenobiotic stress by activating ISR. As we could not comment on all four upstream kinases, due to lack of reliable western blot based data, we could only say that at least for PKR mediated activation of ISR is dependent on FMRP in the lung epithelial cells. Though this study could not delineate the underlined molecular mechanism of FMRP dependent eIF2α phosphorylation in response to stress, but can propose a plausible mechanism that FMRP may physically interact with its binding partner Caprin1 and G3BP1 to facilitate phosphorylation of eIF2α.



Model for the role of FMRP in the regulation of the Integrated Stress Response in the lung. Exposure to xenobiotics such as Naphthalene (Nap) and 9, 10-Phenanthrenequinone (PQ) result in the activation of at least one of four stress-responsive kinases (PKR) and to the induction of Integrated Stress Response Pathway (ISR, outlined in red). Our findings suggest that FMRP has an essential role downstream to PKR phosphorylation (outline in blue).

Chapter- IV: This chapter deals with the alternatively possible mechanisms, which may explain the FMRP phenotype in the lung. First of all, we tried to probe the FMRP and Superoxide dismutase 1 (SOD) interaction in lung in order to understand the role of FMRP dependent SOD1 translation in the susceptibility of FMRP deficient cells. SOD1 is one of the most important enzyme, which regulates the redox balance in lung. SOD1 converts free oxygen radicals in to oxygen molecule and hydrogen peroxide and further degrades hydrogen peroxide to water and hydrogen molecule. Severe lung phenotypes and pathological conditions are associated with mutation of SOD1 gene or loss of function of the protein. A study in mouse brain by Bechara et al, showed FMRP binds with SOD1 mRNA and facilitates its translation. In absence of FMRP there is decreased SOD1 expression in mouse brain.

In light of this study, we tried to probe the role of FMRP in regulating SOD1 expression in lung. Though we could recapitulate the deficiency of SOD1 in mouse brain, but could not detect any significant difference of SOD1 expression in the lung of FMRP deficient mice. This data primarily suggested that SOD1 translation is not dependent on FMRP in mouse lung. But further study during naphthalene insult revealed a possible injury dependent role of FMRP in the upregulation of SOD1 post injury. Studies in mouse cell line with naphthalene and 9, 10 PQ suggests that post injury SOD1 upregulation could be fully or partially dependent on FMRP according to the type of stressors or stress level. But, studies in BEAS2B cell line showed that post injury SOD1 upregulation is not dependent on FMRP in this human bronchial cell line. Altogether the data allow us to conclude that FMRP may regulate post injury SOD1 expression in a context specific way and may not explain the phenotype of FMRP in lung.

Next, we tried to probe the role of FMRP in stress granule biogenesis under the experimental condition. When we treated the cells with arsenite, we observed FMRP and Tia1 are forming large granular structures and mostly they are overlapping with each other. In absence of FMRP these granules were largely absent. Though when we treated the cells with Nap/PQ we did not observe such conspicuous granule formation. We found that maximum percentage of intensity is coming from the diffused staining of FMRP and Tia1 in the control cells (Sc treated) and in absence of FMRP the overall intensity of Tia1 is less compared to control. These results suggest that there may not be any stress granule formation under the experimental condition, even in the presence of FMRP. So, FMRP dependent stress granule biogenesis cannot sufficiently explain the phenotype in the lung.

Next, we logically concluded that FMRP dependent activation of DNA damage response during replication stress cannot explain the phenotype in the lung. FMRP was found to be involved in γ-H2AX mediated activation of DNA damage response during replication stress (Alpatov et al, 2014). It was also reported that in absence of FMRP γ-H2AX foci formation was reduced. Though the report also said that γ-H2AX foci formation may not depend on FMRP in case of certain stressors like γ-irradiation. Under our experimental condition it was found that γ-H2AX foci formation was not perturbed, in fact increased in

absence of FMRP. This section also dealt with the fact that lung epithelial cells and C22 cells under experimental condition should not acquire any replication stress as they were largely non-mitotic. In case of other two cell lines (BEAS2B and A549) however this argue does not apply but increased γ-H2AX foci formation in absence of FMRP clearly tells that perturbation of DNA damage response unable to explain the susceptibility of FMRP deficient pulmonary epithelial cells to xenobiotic stress.

Taken together, in this chapter it was concluded that FMRP dependent actuation of ISR can only explain the underlined mechanism of susceptibility of FMRP deficient pulmonary epithelial cells to the xenoiotic stress across species and stressor.

LIST OF PUBLICATIONS

1. Deblina Sain Basu, Rital Bhavsar, Imtiyaz Gulami, Saraswati Chavda, Sai Manoz Lingamallu, Ravi Muddashetty, Chandrakanth Veeranna, Sumantra Chattarji, Rajesh Thimmulappa, Aditi Bhattacharya, Arjun Guha. FMRP protects the lung from xenobiotic stress by facilitating the Integrated Stress Response. J Cell Sci. 2022 Mar 23; DOI: 10.1242/jcs.258652c

2. Amrutha Kizhedathu, Piyush Chhajed, Lahari Yamala, Deblina Sain Basu, Tina Mukherjee, Vinothkumar Kutti Ragunath and Arjun Guha. Duox generated reactive oxygen species activate ATR/Chk1 to induce G2 arrest in Drosophila tracheoblasts. Elife, 2021 Oct 8; 10:e68636, doi: 10.7554/eLife.68636

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